PASSIVE TRANSFER OF TRANSPLANTATION IMMUNITY

IV. Transplantation Antibody from Extracts of Sensitized Lymphoid Cells*,‡

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A serum antibody capable of destroying a solid homograft has rarely been found. Stetson and Demopoulos (1) and Kretschmer and Pérez-Tamayo (2) reported successful homograft rejection by placing, in and around the test graft bed, serum from animals sensitized by the homograft tissues. Voisin and Maurer (3), Stetson and Jensen (4), and Steinmuller (5) achieved similar results by injecting serum from homografted animals at a distance from the test skin. Other investigators have been unable to destroy solid homografts with antisera, but have succeeded in injuring or inhibiting normal or neoplastic cells in suspension (6).

In this paper, we describe the preparation from sensitized lymphoid cells of a "soluble substance" destructive of solid skin homografts at a distance, its specificity, and a partial characterization of its nature.

Materials and Methods

In all experiments A^1 strain mice provided skin and lymphoid tissue to sensitize CBA^1 recipients, and also the test skin homografts for assay of the graft-destroying substance. In one experiment, AKR^1 and C_3H^1 test skin homografts were used to determine specificity of reaction. CBA spleen and cervical, axillary, and foreleg lymph nodes were the source of the graft-rejecting agent.

Preparation of Transplantation Antibody.—A triangular piece of full thickness A skin was sutured into a skin bed prepared in the nuchal region of recipient CBA mice. Two days later, the recipients were injected intraperitoneally with 20 to 25 $\times$ 10^6 A spleen cells (equivalent to $\frac{1}{4}$ of a spleen) contained in 1.0 ml of balanced salt solution (BSS) and 20 per cent polyvinylpyrrolidone (PVP).[2] Ten days after skin grafting, batches of 20 to 40 sensitized

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[1] All mice were purchased from Jackson Memorial Laboratory, Bar Harbor, Maine.

[2] Distributed by Schenley Laboratories Inc., New York, as a 3.5 per cent solution of PVP-macrose.

CBA mice were etherized to death, their spleens and lymph nodes were removed, pooled, and raked into a suspension in BSS-PVP medium. The suspensions were passed through 80 mesh monel gauze and 100 mesh nylon gauze filters and diluted with medium so that the total volume in milliliters equalled the number of sensitized mice. Counts were made of the final suspension and showed on the average about 150×10^6 cells/ml, the contribution of 1 mouse or an animal equivalent (AE). The suspended cells were then disrupted in a sonic oscillator[3] for 20 minutes, by which time no cells were visible in aliquots examined with the light microscope. The resultant homogenate was centrifuged at 3000 RPM for 30 minutes and yielded an opalescent supernatant and a pellet of cellular debris. The pellet, when assayed, was resuspended in a volume equal to that of the supernatant. All manipulations were done at 4°C.

Bioassay.—CBA mice were grafted with full thickness A skin, 1.5 cm on edge, sutured in the upper dorsum. One day later, the material to be tested for transplantation antibody was injected twice daily for 5 days intraperitoneally into lightly etherized CBA recipients. The test graft and surrounding host skin were removed on the 6th day and processed for histologic examination. Scoring was based on the extent of epithelial necrosis in the test skin: complete rejection, 90 per cent or more; partial rejection, between 10 per cent and 90 per cent; no rejection, 10 per cent or less.

Disc Electrophoresis.—Disc electrophoretic analysis of the supernatant from disrupted sensitized lymphoid cells was performed according to the method of Ornstein and Davis (7) in 1 cm in diameter gel columns. Eight bands were visualized and the polyacrylamide gel was sectioned into 8 segments. Each segment was extracted with isotonic saline and the extracts assayed against specific skin homografts.

DEAE Column Chromatography.—The supernatant of sonically disrupted sensitized lymphoid cells was passed through a 2.2 cm in diameter DEAE column according to the method of Peterson and Sober (8) and Fahey *et al.* (9). In one run, a protein fraction was isolated in each sodium phosphate buffer (buffer 1, 0.0175 M; buffer 2, 0.04 M; buffer 3, 0.1 M; and buffer 4, 0.4 M); in another run, 6 fractions were isolated in the 4 buffers as indicated in Table III.

Ammonium Sulfate Precipitation.—To the supernatant of disrupted sensitized lymphoid cells was added saturated ammonium sulfate so that the final concentration of the salt was 37 per cent. The precipitate was separated from the supernatant, redissolved in isotonic saline, dialyzed against saline for 3 days until free of ammonium sulfate, and adjusted in volume to equal in milliliters the number of mice which provided the lymphoid tissues. The supernatant was also dialyzed against isotonic saline for 3 days and brought to the same volume as the dissolved precipitate.

Total Body Irradiation.—In one experiment, CBA mice carrying test skin homografts were given 650 roentgens total body irradiation just prior to grafting. The irradiation was done by two opposing 220 kv Picker x-ray machines, 175 cm apart, with a leucite animal container midway between them. They were operated at 210 kv, 15 ma, with an inherent filtration of ¼ mm copper and 1 mm aluminum. Together the machines delivered a dose of 55 r per minute in the middle of a paraffin phantom placed in the leucite animal container.

RESULTS

Skin Homograft Rejection by Soluble Substance.—The supernatant of sensitized lymphoid cells destroyed by sonic vibration was found to be effective in rejecting homologous skin grafts 6 days after placement (see Table I). Eighteen of 21

[3] Magnetostrictive oscillator, 200 watt, 10 kc, Raytheon Manufacturing Company, Waltham, Massachusetts.

test grafts were completely destroyed in 6 days and the remaining 3 were partially destroyed. The sediment of the disrupted sensitized lymphoid cells was ineffective; of 21 test grafts there were no complete rejections and 6 partial rejections. The supernatant and the sediment of non-sensitized lymphoid cells, prepared in the same way as those from sensitized cells, were assayed in 16 test grafts; there were no complete and 4 partial rejections.

TABLE I

Skin Homograft Rejection Scores after Transfer of Sonicated Lymphoid Cell Extracts

No. of recipients	Material injected	Dose* per day	Microscopic scores of test skin homografts		
			No rejection	Partial rejection	Complete rejection
		Sensitized lymphoid cells			
4	Supernatant	3 AE‡	0	0	4
9	Supernatant	1 AE	0	1	8
8	Supernatant	½ AE	0	2	6
4	Sediment	3 AE	2	2	0
9	Sediment	1 AE	6	3	0
8	Sediment	½ AE	7	1	0
		Non-sensitized lymphoid cells			
3	Supernatant	3 AE	2	1	0
5	Supernatant	1 AE	3	2	0
3	Sediment	3 AE	3	0	0
5	Sediment	1 AE	4	1	0
		Sensitized lymphoid cells			
5	Supernatant	1 AE	0	1	4
4	Supernatant	½ AE	0	0	4
5	Supernatant	¼ AE	0	2	3
5	Supernatant	⅛ AE	0	2	3
5	Supernatant	1/16 AE	0	1	4

* 5 daily intraperitoneal injections.

‡ Animal equivalent or approximately 1.5×10^8 lymphoid cells.

An attempt to get some quantitative information in terms of animal equivalents (AE) is presented in Table I, lower third. It was possible to destroy completely 4 of 5 test homografts with as little as 1/16 of an animal equivalent, injected daily for 5 days. In terms of cells, the daily dose was equivalent to material obtainable from 10×10^6 sensitized lymphoid cells. It is not meant to suggest that the actual number of cells capable of making or carrying transplantation antibody was known, but that the number of effective cells was within this figure.

Specificity of Transplantation Antibody.—CBA mice were grafted with both an A skin graft and AKR or C_3H skin (see Table II). One animal equivalent of supernatant from lymphoid cells sensitized to A strain tissues was injected intraperitoneally every day for 5 days. Of 13 specific A test grafts, 10 were completely rejected, and 3 partially rejected; of 13 non-specific AKR and C_3H test grafts, there were no complete rejections and 3 partial rejections.

TABLE II

Specificity of Supernatant of Sonicated Lymphoid Cell Extracts

No. of Recipients	Dose* per day	Test graft	Microscopic scores of test skin homografts		
			No rejection	Partial rejection	Complete rejection
7	1 AE‡	Specific A	0	1	6
		Non-specific AKR	5	2	0
6	1 AE	Specific A	0	2	4
		Non-specific C_3H	5	1	0

* 5 daily intraperitoneal injections.

‡ Animal equivalent or approximately 1.5×10^8 lymphoid cells.

TABLE III

Skin Homograft Rejection Scores after Transfer of Supernatant of Sonicated Lymphoid Cell Extracts

No. of recipients	Dose* per day	Microscopic scores of test skin homografts		
		No rejection	Partial rejection	Complete rejection
A. 650 r total body irradiation to recipients				
7	1 AE‡	1	2	4
8	½ AE	2	2	4
B. Supernatant heated to 60°C for 30 min.				
6	1 AE	0	3	3
4	½ AE	0	1	3

* 5 daily intraperitoneal injections.

‡ Animal equivalent or approximately 1.5×10^8 lymphoid cells.

Transferred Antigen and Transfer Factor.—It was possible that the accelerated destruction of solid skin homografts by the supernatant of disrupted sensitized lymphoid cells was due to the presence of an antigen craried over from the A strain–sensitizing tissue, or due to a transfer factor (6) which prepared the host

to destroy the test skins. A probe at this problem was made. CBA mice were given 650 r total body irradiation just prior to grafting with A strain skin. (This amount of total body irradiation prolonged second set and first set graft survival 4 and 6 days respectively.) One day later, the supernatant of sonically destroyed

TABLE IV

Skin Homograft Rejection Scores after Transfer of Fractions of Sonicated Lymphoid Cell Extracts

No. of recipients	Material injected	Dose* per day	Microscopic scores of test skin homografts		
			No rejection	Partial rejection	Complete rejection
		Disc electrophoresis			
4	Segment 1	1 AE‡	0	1	3
4	Segment 2	1 AE	2	2	0
4	Segment 3	1 AE	4	0	0
4	Segment 4	1 AE	4	0	0
4	Segment 5	1 AE	4	0	0
4	Segment 6	1 AE	3	1	0
4	Segment 7	1 AE	4	0	0
4	Segment 8	1 AE	4	0	0
		DEAE column chromatography			
5	Buffer 1	1 AE	0	2	3
5	Buffer 2	1 AE	3	1	1
5	Buffer 3	1 AE	2	2	1
5	Buffer 4	1 AE	4	1	0
		Repeat column chromatography			
5	Buffer 1	1 AE	0	1	4
5	Buffer 2a	1 AE	4	1	0
5	Buffer 2b	1 AE	3	2	0
5	Buffer 3	1 AE	1	3	1
5	Buffer 4a	1 AE	4	1	0
5	Buffer 4b	1 AE	3	2	0
		37 per cent ammonium sulfate			
6	Precipitate	1 AE	0	3	3
6	Supernatant	1 AE	4	2	0

* 5 daily intraperitoneal injections.

‡ Animal equivalent or approximately 1.5×10^8 lymphoid cells.

sensitized lymphoid cells was injected into the CBA mice and repeated again for a total of 5 daily injections. If homograft rejection was the result of a secondary or a primary immunization by a transferred antigen or of a transfer factor, the irradiation should have altered the host so that the survival of the test skins would have been prolonged.

Of 15 test skin grafts on irradiated recipients, 8 were completely rejected at 6 days and 4 partially rejected. There was no prolongation of test graft survival (Table III *A*).

Thermostability.—Supernatant from sensitized lymphoid cells was heated to 60°C for 30 minutes and assayed. As seen in Table III *B*, 6 of 10 grafts were completely and 4 partially destroyed.

Characterization of Transplantation Antibody.—Some physiochemical characteristics of the effective material in the supernatant of disrupted sensitized lymphoid cells are shown in Table IV. By disc electrophoresis, the effective soluble substance was present in the first segment which included the least mobile band. By DEAE column chromatography, active material was present in the first buffer containing the lowest concentration of salt and the highest pH. With ammonium sulfate precipitation, the effective substance was recovered in the precipitate produced by 37 per cent salt concentration and the remaining supernatant displayed no graft-rejecting activity. With these 3 procedures, then, the graft-rejecting agent behaved like a gamma globulin.

DISCUSSION

A "soluble substance" has been obtained from lymphoid cells sensitized by homologous tissues which was capable of destroying a solid skin homograft within 6 days; *i.e.*, in accelerated second set time. The substance was effective at a distance; it rejected the test skin when given intraperitoneally daily for 5 days. It was specific in that an extract derived from non-sensitized cells did not destroy a skin graft within 6 days and the substance derived from sensitized cells rejected the sensitizing A strain skin graft, but did not reject non-specific AKR or C_3H strain grafts in this time. Its behavior in disc electrophoresis, in DEAE chromatography, after heating to 60°C for 30 minutes, and with ammonium sulfate precipitation was similar to that of gamma globulin. These characteristics have suggested an antibody which was initmately associated with lymphoid cells. Parenthetically, sera from CBA mice which were a source of sensitized lymphoid cells were incapable of destroying test grafts in volumes equivalent to those of soluble substance which did reject skin homografts.

The supernatant obtained from low speed centrifugation of sonicated sensitized lymphoid cells undoubtedly contained subcellular particulate matter. In this study it was not determined whether the graft-destroying agent was part of such particulate matter or was truly soluble. We are currently analyzing the immunologic and ultracentrifugal characteristics of soluble substance.

It was unlikely that the supernatant of disrupted sensitized lymphoid cells contained either an activated antigen or transfer factor which prepared the host to reject test homografts in accelerated fashion. 650 r total body irradiation did not influence the complete rejection of test skins by the soluble material, although this amount of total body irradiation prolonged first set and second set graft survival 6 and 4 days respectively.

There have been numerous reports from different groups of investigators attempting to demonstrate that serum antibodies, cell bound antibodies, or both, were responsible agents in homograft rejection (6). In several species solid skin grafts have been destroyed by serum from immunized hosts, injected either locally or at a distance (2–5). Others have repeatedly shown that serum from tissue-sensitized animals contained cytotoxic and hemagglutinating antibodies when tested against cell suspensions of normal or neoplastic tissues (6). We are unable, at present, to relate the antibody described in this paper to these various serum antibodies.

Nor is it possible to state how solid homograft rejection was achieved *in vivo*. In earlier experiments using sensitized lymphoid cells labeled with H_3-thymidine, the tagged cells were rarely found in the rejection site at any time interval from 1 to 6 days after grafting, despite the effectiveness of adoptive immunity (10, 11). These observations suggested that perhaps the presence of the cells carrying transplantation immunity in the graft bed was not necessary for graft destruction and that an antibody could be made and discharged at a distance from the target. The fact that an antibody was extracted from sensitized lymphoid cells which destroyed a test graft in accelerated fashion lent support to this conclusion. But our observations, past and present, have not disclosed how or whether such an effective antibody circulated and reached its target, either free at such low levels as to be undetectable, or bound to or incorporated within certain cell types.

SUMMARY

Passive transfer of homograft immunity was successfully achieved by injection of the supernatant obtained from tissue-sensitized lymphoid cells disrupted by sonic vibration. The effective substance destroyed specific skin homografts within 6 days but did not reject non-specific skin grafts in this time. No evidence of transferred antigen or of transfer factor was found when the effective material was passed to irradiated recipients carrying test grafts. By a variety of physiochemical procedures the "soluble substance" behaved like a gamma globulin and was considered to be a transplantation antibody.

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